

Differential atrial versus ventricular ANKRD1 gene expression is oppositely regulated at diastolic heart failure

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Abstract Diastolic heart failure (DHF) was produced in 6-day-old piglets by intravenous administration of Doxorubicin, and ANKRD1 protein and mRNA levels were determined in atrial (A) and ventricular (V) chambers of failing vs control hearts. In controls, ANKRD1 showed a left–right (L–R) asymmetric distribution with protein levels 2-fold higher in the LA as compared to the RA, and 8-fold higher in the LV than the RV. In failing hearts, ANKRD1 levels were augmented about 2-fold in each ventricle but equally reduced in both atria as compared to controls. ANKRD1 downregulation in atria is discussed as a process associated with advanced DHF.
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1. Introduction

The cardiac ankyrin repeat domain 1 protein (ANKRD1), an early marker of cardiac cell differentiation, is expressed at high levels in fetal, neonatal and adult heart [1–3]. As a result of the work of many investigators, there is increasing evidence to suspect an involvement of ANKRD1 in a broad range of cardiac muscle disorders, including heart failure (HF). Accordingly, ANKRD1 has been identified as a gene that is strongly upregulated in left ventricle (LV)-myocardium in both animal models [3–6] and patients [7,8] at HF secondary to various cardiomyopathic conditions. Although the results as reported are insufficient to causally link the induction of ANKRD1 to the decreased contractility of LV myocardium *in vivo*, they do point to a potentially important role of ANKRD1 in downstream events associated with LV-myocardium response to HF conditions [8,9].

HF is often considered to be a consequence of decreased contractility and/or reduced ejection fraction of the LV. However, a large number of patients with HF symptoms have a preserved systolic function but abnormal diastolic function, a syndrome which is defined as diastolic heart failure (DHF) [10–13]. Recent clinical evaluations highlight the important role of the left atrium (LA) in pathogenesis of DHF [14,15]. To the best of our knowledge, cardiac ANKRD1 expression has not yet been studied at DHF. Moreover, alterations in the ANKRD1 protein distribution in atrial chambers in response to HF-conditions have not been analyzed in detail [7,16] and have received no further consideration.

The goals of our experiments, therefore, were to develop a DHF-like model in neonatal piglets and to determine a fingerprint of ANKRD1 gene expression throughout the heart, accounting for region- and disease-specific aspects. We have previously shown a differential response of pig neonatal heart to increasing doses of cardiotoxic antibiotic, Doxorubicin (Dox) [3]. In the present work, neonatal piglets exposed to a single dose (1.5 mg/kg) of Dox developed a biventricular diastolic dysfunction resulting in DHF. In failing hearts, ANKRD1 protein and transcript content was significantly augmented in ventricles, but reduced in atria. Taken together, the results strongly suggest that: (1) distinct ANKRD1 regulatory programs are operative in ventricular and atrial myocardium at DHF; and (2) ANKRD1 downregulation in atria is a differential sign of DHF, since acute systolic dysfunction resulted in ANKRD1 upregulation in both atria and ventricles.

2. Materials and methods

2.1. Animals and experimental procedures

All experimental procedures were carried out in accordance with the European Commission Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. Six-day-old piglets were randomized in two groups assigned to receive a single i.v. injection of isotonic saline (PBS) or 1.5 mg/kg of Dox (Sigma) as described in [3]. On day 24 after injection, cardiac output (CO), global end-diastolic volume (GEDV), and extravascular lung water (EVLW) were monitored in close-chest piglets by PiCCO device (Pulsion AG, Germany) in accordance with the manufacturer's recommendations. The measurements of ventricular end-systolic (LV/RVESP) and end-diastolic pressure (LV/RVEDP) were performed in open-chest piglets using a Dräger UM 3.1 pressure transducer and a recording device (Drägerwerk AG, Germany) as described in [17]. Then piglets were euthanized to harvest cardiac tissue for measurement of heart weight, LV/RV free wall thickness, LA size, and for RNA and protein isolation, and fixation [3,18]. Endocardial samples with or without Purkinje cells were isolated from the LV of control piglets under a Nikon SMZ-2 T microscope (Fig. 1G).

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Abbreviations: ANKRD1, ankyrin repeat domain 1 protein; HF, heart failure; DHF, diastolic heart failure; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; Dox, doxorubicin; CO, cardiac output; GEDV, global end-diastolic volume; EVLW, extravascular lung water; ESP, end-systolic pressure; EDP, end-diastolic pressure; CASQ2, calsequestrin 2; BNP, brain natriuretic peptide; RPL19, ribosomal protein L19

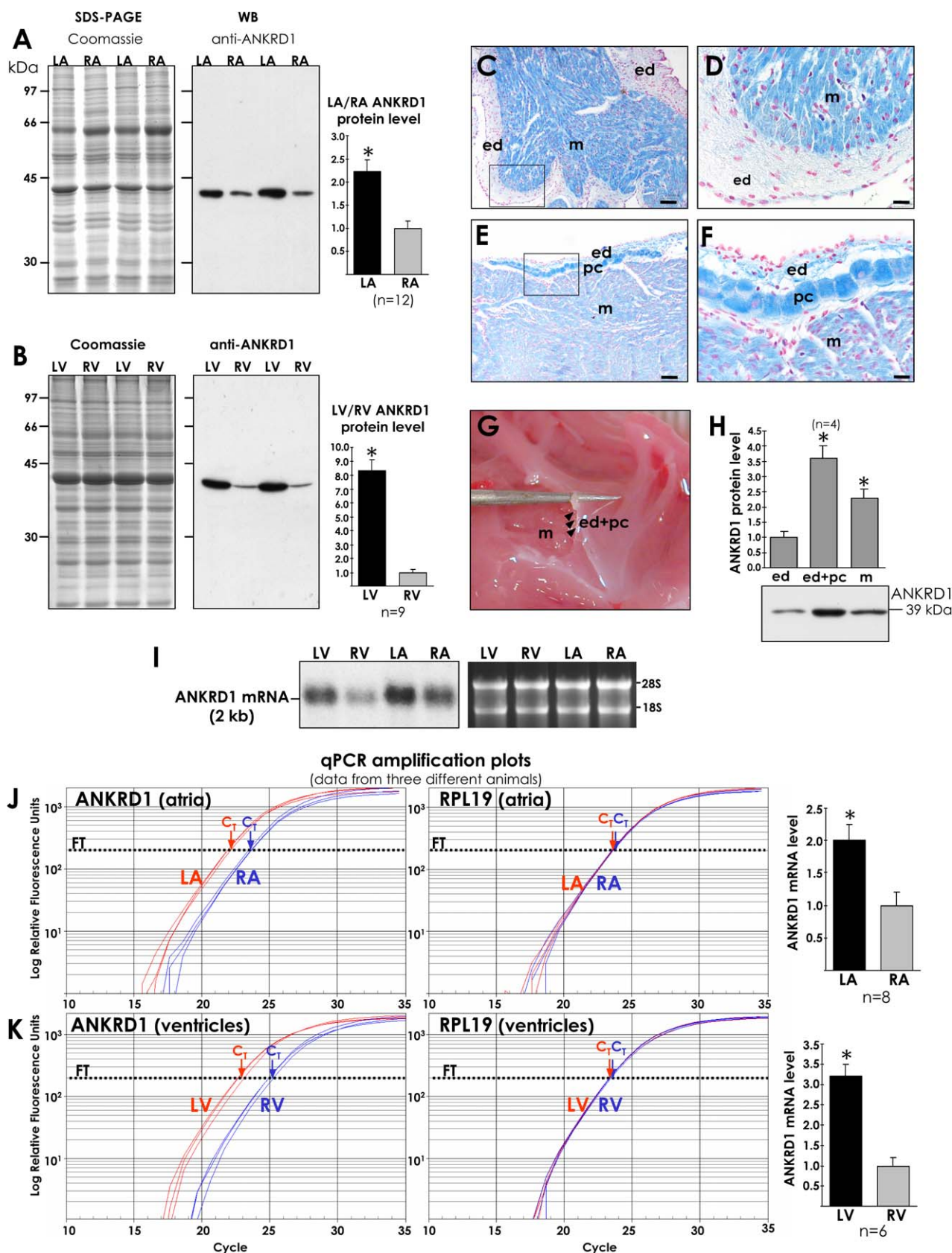


Fig. 1. ANKRD1 expression in four cardiac chambers of control piglets. A and B, Western blot (WB) analysis of ANKRD1 protein levels in atria and ventricles, respectively. C, D and E, F, ANKRD1 immunoreactivity (blue staining) on LA and LV sections, respectively. ed, endocardium; pc, Purkinje cells; m, myocardium. Bar: C,E 68 μ m; D,F 17 μ m. G, sampling of LV endocardium (ed), Purkinje cell-containing endocardium (ed + pc), and myocardium (m). H, ANKRD1 levels in ed, ed + pc, and m. I, Northern blot hybridization signals for ANKRD1, and control of RNA loading. J, K, qPCR analysis of ANKRD1 mRNA levels (normalized to the internal standard RPL19) in ventricles and atria, respectively. C_T , cycle threshold; FT, fluorescence threshold. * $P < 0.05$.

2.2. Quantitative immunoblotting

SDS–polyacrylamide gel electrophoresis and quantitative Western immunoblotting were performed as described in [3,19] using recombinant full-length porcine ANKRD1 as a standard. Briefly, snap-frozen cardiac-tissue samples were pulverized and lysed in 2× SDS-sample buffer (10 µl/mg of frozen powder) supplemented with complete protease inhibitor cocktail (Roche). Resulting extracts (range of 0.2–8 µg of total protein) were SDS-electrophoresed and transferred to PVDF membrane (Hybond-P, Amersham Biosciences). The replicas were incubated with rabbit anti-pig ANKRD1 antibodies [3] and detected using HRP-conjugated anti-rabbit IgG (Sigma) and the Super-Signal West Pico chemiluminescent substrate (Pierce Biotechnology) according to the manufacturer's instructions. The blots were re-probed with anti-canine cardiac calsequestrin 2 (CASQ2) antibodies (Abcam) as an additional control for loading. Quantification of Western blots was obtained by using a Bio-Rad GS700 densitometer with Quantity One software.

2.3. Immunohistochemistry

Immunodetection of ANKRD1 on paraformaldehyde-fixed, paraffin-embedded sections was performed as described in [18]. Serial sections of control and failing heart tissues were incubated with primary rabbit anti-ANKRD1 immuno-affinity purified antibodies. Then the sections were assayed using the Vectastain ABC-AP kit (Vector Laboratories) in accordance with the manufacturer's recommendations and counterstained with nuclear fast red. Substitution of the primary antibodies with anti-ANKRD1 antibodies pre-adsorbed by purified full-length porcine ANKRD1 was included in negative controls.

2.4. Northern blot

Total RNA was isolated from snap-frozen cardiac samples using RNeasy Midi columns (Qiagen) according to the manufacturer's protocol. RNA (5 µg/run) was separated by 1.2% formaldehyde–agarose gel electrophoresis and transferred to a nylon Hybond-N membrane (Amersham Biosciences). The pig ANKRD1 full-length coding sequence labeled with [α -³²P]dCTP was used as a probe. Blots were prehybridized, hybridized, washed, and developed as described previously [3].

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (qPCR)

Two-step qPCR [20] was performed on a Bio-Rad IQ5 detection system with SYBR Green mix. Non-template and non-RT RNA template reactions were used as negative controls. Primers for each porcine gene located in different exons were as follows: ANKRD1 (207-5'-TAG-AAGCTGGAGCCCCAGATTG-3' and 208-5'-GCAACTTATCCCC-GAGCACTGA-3'), brain natriuretic peptide (BNP: 82-5'-GCTCCT-GCTCCTGTTCTTGCA-3' and 251-5'-GGTCCAGCAGCTCCTG-TATC-3'), ribosomal protein L19 (RPL19: 64-5'-CTGCTCAGAA-GATACCGTGAAT-3' and 206-5'-GCTTGTGGATGTGCTCCATGA-3'). RPL19 was used as an internal standard, because its expression was not different throughout cardiac chambers nor between control and Dox-treated hearts [3,21]. Each primer pair used yielded a single peak of dissociation on the melting curve and a single band with the expected size on agarose gel. Identity of the PCR products was confirmed by sequencing. All reactions were run in duplicate in at least two independent experiments. Normalized gene expression analyses were performed with Bio-Rad IQ5 software.

2.6. Statistics

The data are presented as means \pm S.E.M. Comparisons were made using paired Student's *t*-test. Probability values of *P* < 0.05 were considered statistically significant.

3. Results

3.1. Dox-injected neonatal piglets develop the features of human DHF

Ventricular-chamber performance *in vivo* and cardiac morphology *ex vivo* were analyzed in piglets 24 days after Dox administration (Table 1). LV/RVESP was slightly lower,

whereas LV/RVEDP (an accepted marker for ventricular compliance) was significantly higher in Dox-injected compared to PBS-injected piglets. These findings are further corroborated by pulmonary congestion (elevated EVLW indexes) indicating severe diastolic dysfunction (with preserved CO) in DHF-piglets. Failing hearts displayed little change in LV/RV free-wall thickness. In Dox-injected piglets, BNP (an accepted cardiac stretch response marker) mRNA levels increased in both ventricles and in the RA, remaining unchanged in the LA.

3.2. ANKRD1 displays left vs right and atrial vs ventricular expression patterns in sham piglets

In sham piglets, ANKRD1 showed a striking asymmetric L–R distribution with protein levels 2-fold higher in the LA as compared to the RA, and protein content enriched 8-fold in the LV over the RV (Figs. 1A and B). The ANKRD1 mRNA expression was coincidentally enhanced 2-fold in the LA vs RA while only 3-fold higher in the LV as compared to the RV (Figs. 1J and K). Further, ANKRD1 protein and mRNA were more abundant in the LA vs LV and in the RA vs RV as revealed by Western blot and Northern blot hybridization, respectively (Figs. 1I and 2E, control).

In both LA and RA, ANKRD1 immunoreactivity was predominantly expressed throughout the entire atrial myocardium and was low or undetectable in endo- and epicardial layers (Figs. 1C and D). We were the first to demonstrate the localization of ANKRD1 in ventricular Purkinje cells [18]. In this work, immunohistochemical analysis yielded strong ANKRD1 signals in ventricular Purkinje cells and, though to a less extent, also in working myocytes (Figs. 1E and F). As revealed by Western blot, the ANKRD1 protein content in the Purkinje cell-containing samples was about 2-fold higher as compared to that in subjacent working myocytes (Figs. 1G and H).

3.3. ANKRD1 shows opposite patterns at DHF being upregulated in ventricles and downregulated in atria

In DHF hearts, the ANKRD1 protein content was significantly augmented in both LV (2.3 \pm 0.4-fold vs sham) and RV (3.1 \pm 0.5-fold vs sham), but reduced in the LA (0.4 \pm 0.1-fold vs sham) and RA (0.5 \pm 0.1-fold vs sham) (Fig. 2A–D). In contrast to ANKRD1, levels of the MLC2a, cardiac α -actin, β -myosin heavy chain, were found to be simi-

Table 1
Cardiac parameters of control and Dox-injected piglets

Parameter	Control (PBS)	Dox (1.5 mg/kg)
Number of animals	12	12
Heart/body ratio, $\times 1000$	6.6 \pm 0.3	6.4 \pm 0.2
LV free wall thickness (mm)	9.7 \pm 0.5	11.1 \pm 0.5
RV free wall thickness (mm)	2.7 \pm 0.7	3.8 \pm 0.6
LV end-systolic pressure (mm Hg)	80.6 \pm 5.1	67.1 \pm 3.3*
LV end-diastolic pressure (mm Hg)	3.8 \pm 1.1	8.5 \pm 1.3*
RV end-systolic pressure (mm Hg)	18.0 \pm 1.3	16.9 \pm 0.9
RV end-diastolic pressure (mm Hg)	3.3 \pm 1.0	6.1 \pm 1.1*
Cardiac output index (L/min/m ²)	3.4 \pm 0.4	3.4 \pm 0.6
Extravascular lung water (ml/kg)	57.2 \pm 8.4	101.3 \pm 31.5*
LV BNP mRNA, relative value	1.0 \pm 0.1	5.1 \pm 0.3*
RV BNP mRNA, relative value	1.0 \pm 0.2	6.3 \pm 0.5*
LA BNP mRNA, relative value	1.0 \pm 0.2	1.3 \pm 0.4
RA BNP mRNA, relative value	1.0 \pm 0.3	2.4 \pm 0.6*

**P* < 0.05.

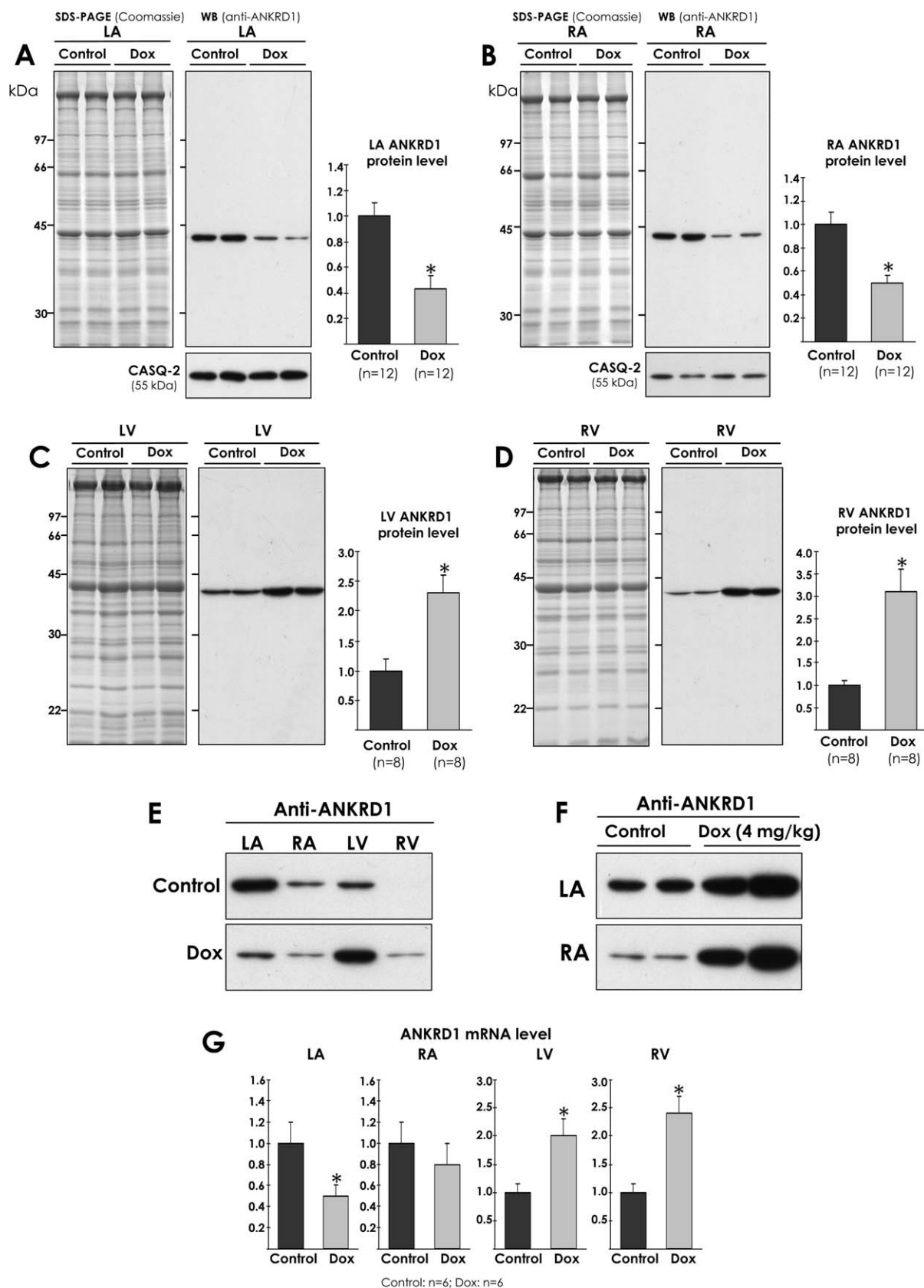


Fig. 2. ANKRD1 expression in four cardiac chambers of Dox-injected vs control piglets. A, B, C, and D, Western blot analysis of ANKRD1 protein levels in LA, RA, LV, and RV, respectively. CASQ2 – internal control of protein loading. E, Western blot analysis comparing ANKRD1 expression levels in four cardiac chambers of control vs 1.5 mg/kg Dox-injected piglets. F, Western blot analysis of ANKRD1 levels in the LA and RA of control and 4 mg/kg Dox-injected piglets. G, qPCR analysis of ANKRD1 mRNA levels (normalized to RPL19) in LA, RA, LV, and RV of control and Dox-injected piglets. * $P < 0.05$.

lar in failing and non-failing atria. Immunohistochemical examination revealed a stronger ANKRD1 immunostaining of working myocytes in failing ventricles, whereas failing atrial myocardium staining was weaker as compared to respective tissue-sections in controls (not shown).

Dox-induced DHF led to upregulation of mRNA transcripts for ANKRD1 in both LV and RV to an extent similar to that of ANKRD1 protein enrichment in each failing ventricle (i.e., about 2-fold vs sham; see Fig. 2G). However, in the failing atrial myocardium, whereas ANKRD1 protein was downregulated in both atria, the ANKRD1 mRNA was downregulated in the LA (2.1 ± 0.5 -fold vs sham) but insignificantly reduced in the RA (Fig. 2G). It is possible that in DHF hearts the ANKRD1 protein is translated with a lower efficiency in the RA as compared to the LA.

Thus, in our model of DHF-like syndrome the ANKRD1 protein concentration equally increased in both ventricles, whereas in both atria its concentration showed a sharp reduction. Owing to these opposite expression kinetics, the normal LA-predominant pattern of ANKRD1 distribution was conversely changed to a LV-predominant one in failing hearts (Fig. 2E).

To determine whether ANKRD1 downregulation in atria was an advanced event of DHF and not a consequence of unspecific Dox effects, we screened ANKRD1 distribution in atria of neonatal piglets that received a dose of Dox (i.e., 4.0 mg/kg) about 3-fold higher than that used in the present work. Dox, at a dose of 4 mg/kg, caused the rapid development of LV myocardial dysfunction and acute systolic HF accompanied by a marked ANKRD1 and BNP upregulation in both ventricles [3]. Western blot analysis demonstrated that in these animals ANKRD1 was dramatically upregulated in both failing atria, in sharp contrast to its downregulation in atrial myocardium in our model of DHF (Fig. 2F).

4. Discussion

Utilizing a newly developed model of DHF-like syndrome in neonatal piglets, this study examines, for the first time, the relationship between changes in expression of the ANKRD1 gene in four cardiac chambers and pathophysiological abnormalities of ventricular/atrial functions. In humans with DHF, LV relaxation is impaired, which increases LV and LA diastolic pressure which, in turn, results in elevated LA myocardium tension that culminates in enlarged LA. Accordingly, LA size/volume is considered a predictive “morphologic expression” marker of DHF [13,22]. We show here that ANKRD1 is downregulated in the LA of DHF piglets at both the protein and mRNA level. To the best of our knowledge, this is the first report that implicates ANKRD1 as a possible molecular predictor of advanced atrial dysfunction at experimental DHF.

Why ANKRD1 is downregulated in atria and what consequences such downregulation at DHF might have are not clear. Initially, ANKRD1 was isolated as a cardio-restricted binding partner of YB-1 (ubiquitous transcription factor), and so ANKRD1 was implicated in the negative regulation of muscle gene expression [1,23]. Thereafter, ANKRD1 was identified as a binding partner of sarcomeric proteins, myopalladin [24] and titin [25] suggesting a role of ANKRD1 in sarco-

meric integrity and myofibrillar stretch sensing, respectively. More recently, ANKRD1 has also been shown to interact with cardiac CASQ2 [18], desmin, and itself [26]. This *in vitro* data indicates that ANKRD1 may be a multifaceted adaptor polypeptide capable of binding various muscle-associated proteins, and accordingly ANKRD1 could be simultaneously implicated in diverse processes in the cardiomyocyte that adds an additional layer of complexity to the study of ANKRD1 functions *in vivo*.

Further, as shown in this work, the normal postnatal heart is characterized by clear regional differences in ANKRD1 expression, such as L vs R and A vs V. This is consistent with our previous work demonstrating a similar ANKRD1 mRNA and protein fingerprint in the heart of newborn piglets [3]. If postnatal ANKRD1 expression is chamber-dependent, then one would expect that altering its expression in response to cardiac insults could be, at least in part, chamber-specific [3,7]. Under our experimental conditions, the ANKRD1 protein content was equally augmented in both ventricles but equally decreased in both atria indicating, therefore, a different A–V response of postnatal myocardium to Dox-induced DHF. Of note, in spite of this opposite A–V response, the asymmetric L vs R pattern of ANKRD1 expression persisted in failing hearts.

HF can result from predominant LV, RV or biventricular dysfunction, and cardiac regional ANKRD1 expression may be affected to a different degree depending on pathophysiological states [3,7]. In our DHF-like model, elevated LV/RVEDP could lead to a state of biventricular myocardium stretch until the ventricles become so stiff that the easily distensible thin-walled atrial myocardium fails to increase diastolic filling pressure. At advanced stages, chronic stretch of the ventricular muscle fibers can result in significant ANKRD1 upregulation in each ventricle [25,27,28], whereas an imbalance between the increase in contractile demand (due to excessive atrial pressure) and the contractile capacity of atrial myocytes [13] might provoke ANKRD1 downregulation in atria.

Insight into ANKRD1 downregulation might come from studies on *in vitro* cultivated heart-derived myocytes exposed to oxidative stress-inducing agents (including Dox). Such agents downregulate ANKRD1 expression probably via induced overexpression of the proapoptotic transcriptional factor, GADD153 [29]. However, in our experiments Dox application (1.5 mg/kg) led to significant ANKRD1 upregulation in both ventricles. Moreover, at higher Dox-doses (4 mg/kg) ANKRD1 was upregulated in both ventricles [3] and atria (this work). Taken together, the results strongly suggest that the opposite A–V ANKRD1 expression kinetics at experimental DHF are associated with different functional alterations of ventricular and atrial myocardium rather than with hypothetical differences in the sensitivity of A and V myocytes to a single Dox-exposure *in vivo*. Decreased ANKRD1 levels in atria could be considered as a differential sign of diastolic dysfunction, because acute systolic HF was shown to be associated with gene upregulation in both atria (Fig. 2F) and ventricles.

With regard to demonstrated biventricular ANKRD1 upregulation at porcine DHF, it is interesting to note that adenoviral-mediated overexpression of ANKRD1 in neonatal rat ventricular myocytes *in vitro* resulted in decreased contractile responsiveness of infected cells to catecholamine or calcium overloads [9].

In conclusion, the ANKRD1 expression mapping presented here provides the first framework for further and more detailed analysis of normal and pathological regulation of the gene in the four-chambered postnatal heart. At present, our findings demonstrate that the DHF-like syndrome is related to reduced ANKRD1 expression in atria (specifically in the LA) and opposite gene upregulation in ventricles.

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